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Original Article

Anti-Leishmanial Activity of *Artemisia persica*, *A. spicigera*, and *A. fragrance* against *Leishmania major*

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Abstract

Background: Neglected tropical diseases (NTDs) like zoonotic cutaneous leishmaniasis (ZCL), is a widespread infectious disease with high mortality and morbidity. Various medications are used for treating the disease, but several side effects and drug resistance have been reported. Herbal medicines are unlimited sources for discovering new medications to treat infectious diseases. We aimed to determine the leishmanicidal activity of three species of Iranian *Artemisia* herbal plant extracts in in-vitro.

Methods: In-vitro anti-leishmanial activity of ethanolic extracts on both pro-mastigotes and amastigotes was determined by using MTT method. IC50, CC50, EC50 and SI were calculated. The study was done in 2019-2020 in Iran University of Medical Sciences, Tehran, Iran.

Results: All of the three *Artemisia* species significantly reduced the number of parasite promastigotes. Among them, *A. persica* had the highest leishmanicidal activity against parasite promastigotes. Cytotoxicity assay elucidated that the *Artemisia* had no toxicity to the host cells, and killed the *L. major* amastigotes very efficiently. By increasing the dose of extracts, the parasite number in both phases (promastigotes and amastigotes) was reduced significantly.

Conclusion: These results indicated satisfactory anti-leishmanial activity of *Artemisia* extracts against ZCL in-vitro. Accordingly, *Artemisia* ethanolic extracts might be considered as a strong, effective and safe herbal compound for clearing the *L. major* with less toxicity to the host macrophages cells. Hence, it may be recognized as an excellent herbal therapy for treating the ZCL.

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Introduction

Leishmaniasis is one of the most common parasitic diseases that usually produce various devastating disorders on the different parts of the body. This infection is one of the most common neglected tropical disease in the world especially in tropical and subtropical regions like Iran and has been categorized into different forms including cutaneous (CL), muco-cutaneous (MCL), and visceral (VL) types which (1-3). CL is the most prevalent form of the infection with approximately 1.5 million new cases diagnosed each year and over 370 million people are at risk of this infection throughout the world, where nearly all cases occur in several countries such as Iran, Afghanistan, Peru, Algeria, Brazil, Syria and Saudi Arabia (4, 5). Zoonotic CL (ZCL) is one of the most common forms of cutaneous lesions that usually produce various skin lesions. The causative agent of ZCL is *Leishmania major*, an intracellular protozoan infection, which is transmitted by a bite of sand flies to mammalian hosts.

Pentavalent antimonial compounds like glucantime and amphotericin B, are used as the first-line and second-line drugs for the treatment of CL, respectively, which have various toxic effects and lead to serious side effects such as liver, heart and biochemical disorders (6-9). Furthermore, to date, *Leishmania* parasites have developed resistance to existing chemical drugs (10, 11). Thus, determination and development of new medicinal agents are essential for alternative treatment. In this regards, natural herbal compounds with anti-leishmanial effects can be very useful, valuable, safe and inexpensive sources of antimicrobial agent (12, 13). Anti-leishmanial effect of some herbal plants has been attributed to presence of the materials such as quinines, terpenes, steroids and flavonoides (14, 15). In herbal medicine, one of the less-studied herbal plants in *Leishmania* infections is *Artemisia* known as "teretkh" in Iranian folk medicine which is ubiquitously present in different parts of Iran, especially in the northern part of it (16). The *Artemisia* genus as an aromatic perennial herb belonging to astraceae family. Among 500 species of *Artemisia* genus which are grown all over the world, 34 species are distributed in Iran (16, 17). Known plants for their potent chemical constituents and their essential oil which are used as a traditional medicine for the treatment of inflammatory and infectious diseases (18-23). *Artemisia* species have several phytochemical constituents like flavonoids, terpenoids and coumarins. In this herbal plant, various activities such as antimicrobial, anti-hepatotoxic, insecticides, anti-inflammatory, and antimalarials effects have been identified (16, 24-27).

To the best of our knowledge, this is the first experiment measuring the main efficacy, toxicity and antileishmanial activity of three major Iranian flora of *Artemisia* including *A. persica*, *A. spicigera*, and *A. fragrance* ethanolic extract against ZCL due to *L. major* in in-vitro condition and calculate the inhibitory concentration (IC50), cell cytotoxicity (CC50), effective concentration (EC50) and selectivity index (SI).

Materials and Methods

Plant material

The aerial parts of different species of *Artemisia* herbal plant were collected at flowering stage in October and November 2016 from Karaj city, Iran and stored in the Iranian Biological Resource Center (Essential Oil and Extract Bank). The study was done in 2019-2020 in the Department of Parasitology and Mycology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran. They identified in the herbarium of Iranian Biological Resource Center (IBRC), Tehran, Iran. After identification of three species of *Artemisia* including *A. persica*, *A. spicigera* and *A. fragrance*

(plant voucher specimens: IBRC P1006575, IBRC P1000158 and IBRC P1000564, respectively), about 25 g of aerial parts (leaves) of each sample was air-dried at room temperature and grounded separately in an electric grinder and powdered using a mixer and then kept in a dark amber-colored glass bottle before extraction.

Herbal extraction

Powdered samples were macerated in ethanol (Merck) as a solvent and kept for 72 h away from light and high temperature. The ethanolic extraction processes of powdered materials of *Artemisia* species were applied according to the protocol as described previously (28). The extracts were sterilized by filtration by means of a membrane filter (0.22 μm). Stock solutions of this extracts were freshly prepared in 500 µl of dimethyl sulfoxide (0.2% DMSO as a solvent control) and then stored at -20 ˚C for evaluation of its anti-leishmanial activity. Serial dilutions of plants extracts (12.5, 25, 50, 100, 200, 400 and 800 µg/ml) and drug control (glucantime: 150 µg/ml) were prepared in RPMI1640 medium.

Parasite culture

Pathogenic Iranian strain of *L. major* (MRHO/IR/75/ER) promastigotes was applied in the current study. *L. major* amastigotes were isolated from BALB/c mice and transformed to the promastigote form (29-31). The current study does not contain any experiment with human participants or animals performed by any of the authors.

MTT assay

To determine the effects of *Artemisia* ethanolic extract on *L. major* promastigotes, amastigotes and macrophages, cell viability was determined using 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) method (29-31). Samples optical density (OD) was measured using an ELISA plate reader (BioTek Company: USA) at 570 nm. The results were evaluated by linear regression of the inhibition percentage (32).

Promastigotes susceptibility assay (IC50)

L. major promastigotes were cultivated in the stationary growth phase in RPMI1640 at 26 °C. For the promastigotes susceptibility tests, a density of 1×10^6 parasite/well was counted in a neubauer chamber by a light microscope and plated in 96-well micro plates. *Artemisia* ethanolic extract susceptibility was determined using colorimetric MTT assay and inhibitory concentration (IC_{50}) that caused a 50% decrease in survival of parasites was calculated (33, 34).

Macrophages culture and cell cytotoxicity assay (CC50)

J774A.1 as a mice macrophage cell line was utilized in the current experiment. Cells were cultured in RPMI-1640 medium (Sigma- Aldrich Chemicals; Germany) with heat-inactivated fetal calf serum (FCS, 10%), supplemented with penicillin and streptomycin (100 µg/ml; pH=7.4). Cell viabilities were measured using colorimetric MTT assay and cytotoxicity concentration (CC_{50}) was determined (35).

Cell line infection and effective concentration assay (EC50)

J774.A1 macrophages (5×10^3 cells) were plated in 96-well culture plate in RPMI1640 medium supplemented with FCS 10%, incubated at 37 ˚C in CO2 5% for 24 h. Then, the stationary growth phase promastigotes of *L. major* were seeded into each well (1:10) and incubated at 37 ˚C in CO_2 5% for 24 h allowing the promastigotes to penetrate and infect the cells. Later, free promastigotes were removed through washing using RPMI1640 medium. The infected macrophages were treated with several increasing concentrations of *Artemisia* extracts as mentioned previously for 48 h at 37 °C in CO2 5%. Finally, infected cell viability was evaluated by MTT assay and results were reported as effective concentra-

tion (EC50) that killed 50% of intracellular *Leishmania* parasites (36).

Selectivity Index determination

The ratio of the obtained CC_{50} value of the cytotoxic concentrations to the obtained EC_{50} value of the antileishmanial activity was determined in order to calculate the *Artemisia* selectivity index (SI) (37). Moreover, SI was calculated for promastigote forms of parasite (SI= CC_{50} Macrophages / IC_{50} Promastigotes) (38). At a time that the SI value is under 10, that compound has an ideal antileishmanial activity. On the other hand, the ideal *Artemisia* compound would be cytotoxic slowly at very high concentrations, and have antileishmanial activity at very low concentrations (higher reported values= greater *Artemisia* activity).

Statistical analysis

CC_{50}, IC_{50}, and EC_{50} calculations and statistical analyses were conducted using Prism 8.0 for Windows (Graphpad Prism, San Diego, CA). The differences between control and treatment groups were measured using analysis of variance (ANOVA), and differences with *P*-values of less than 0.05 were considered statistically significant.

Results

Effects of extracts on promastigotes and inhibitory concentration (IC50) estimation

The IC_{50} of *A. persica*, *A. spicigera*, and *A. fragrance* extracts was evaluated against promastigotes of *L. major* in order to estimate the IC50 values. Three studied species of *Artemisia* ethanolic extracts significantly affected the promastigotes growth at 48 h post incubations (Fig. 1). In this regards, the *Artemisia* extract inhibited the *Leishmania* parasite growth with the IC50 of 51 µg/ml, 200 µg/ml and 400 µl/ml for *A. persica*, *A. spicigera* and *A. fragrance*, respectively. Glucantime (Sanofi Aventis, France) as a positive control, which was evaluated at the concentration of 150 µg/ml, could entirely inhibit the *Leishmania* promastigotes growth.

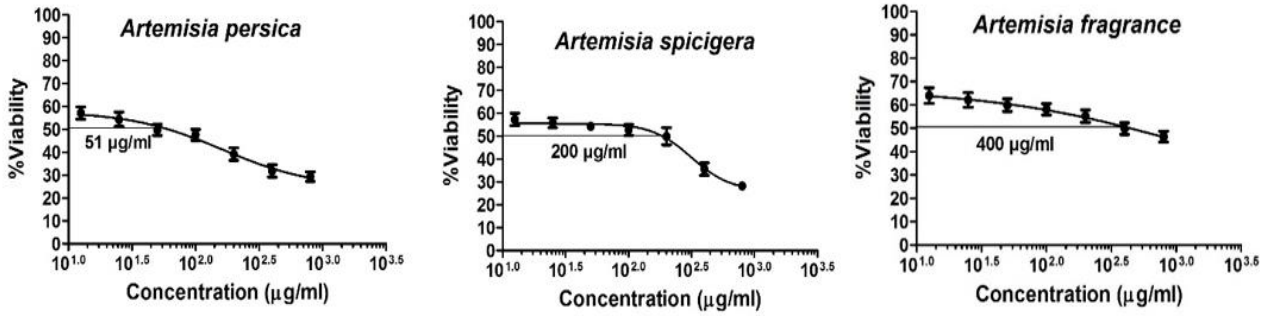

Fig. 1. The inhibitory concentration (IC_{50}) of three species of *Artemisia* ethanolic extracts (increasing concentrations: 12.5, 25, 50, 100, 200, 400 and 800 µg/ml) on the *Leishmania major* promastigotes after 48 h by using MTT assay. All data are reported in this study as mean ± SD of three repeated experiments with same outcomes

Effects of extracts on macrophages and cell cytotoxicity (CC50) estimation

The CC_{50} of different concentrations of *Artemisia* on J774.A1 macrophages was determined at 48 h after incubations and compared with the control groups (Fig. 2). The results showed no significant differences between test groups and the control groups for each *Artemisia* extract ($P<0.05$). The CC50 was calculated to be 518 µg/ml of *A. persica*, 560 µg/ml of *A. spicigera* and 700 µg/ml of *A. fragrance* at 48 h after incubations, respectively (Fig. 2). Ac-

cording to Fig. 2, three species of *Artemisia* ethanolic extracts with various doses in test groups had low cytotoxic effects on uninfected macrophages at 48 hours after incubation and they had been solely toxic for cells at very high concentrations.

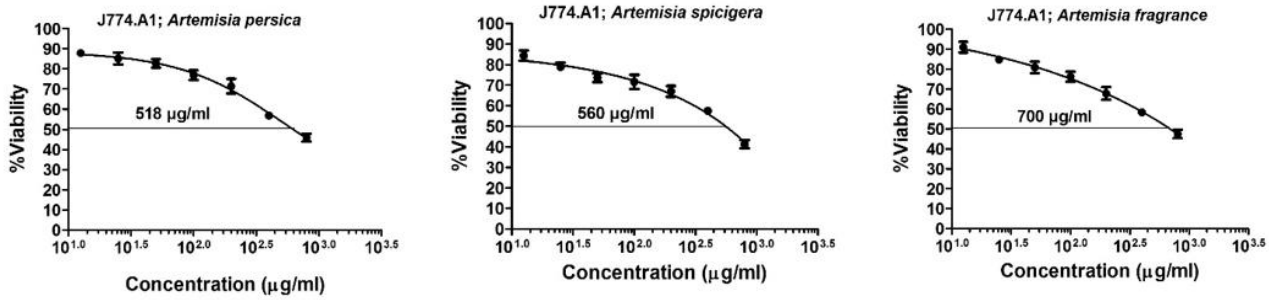

Fig. 2. Cytotoxicity assay (CC_{50}) of three species of *Artemisia* ethanolic extracts (increasing concentrations: 12.5, 25, 50, 100, 200, 400 and 800 µg/ml) on the macrophage cell line (J774.A1) after 48 h by using MTT assay. All data are reported in this study as mean ± SD of three repeated experiments with same outcomes

Effects of extracts on infected macrophages and effective concentrations (EC50) estimation

The EC50 of different concentrations of three *Artemisia* species on infected macrophages with *L. major* was determined at 48 h after incubations and compared with the control groups (Fig. 3). A significant reduction was seen in the percentage of infected cells after 48 h. The EC50 was calculated to be 100 µg/ml of *A. persica*, 100 µg/ml of *A. spicigera*, and 110 µg/ml of *A. fragrance* at 48 h after incubations, respectively (Fig. 3).

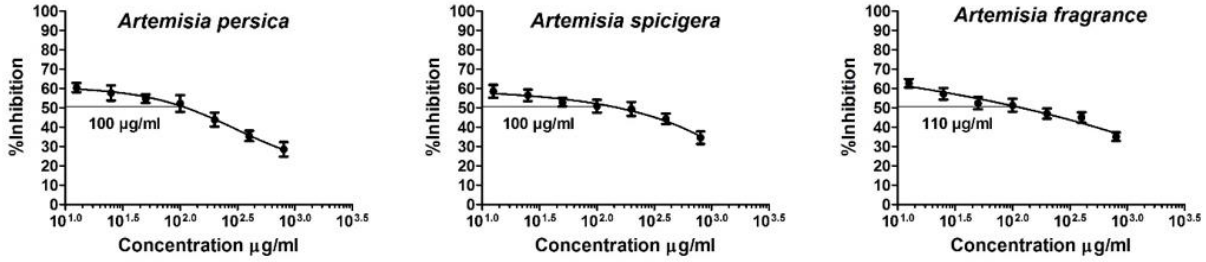

Fig. 3. Effective concentration (EC_{50}) values of three species of *Artemisia* ethanolic extracts (increasing concentrations: 12.5, 25, 50, 100, 200, 400 and 800 µg/ml) on the infected J774.A1 macrophage with *Leishmania major* after 48 h by using MTT assay. All data are reported in this study as mean ± SD of three repeated experiments with same outcomes

Artemisia extracts and selectivity index (SI) estimation

Three species of *Artemisia* extracts were active against the amastigotes of *L. major* with a suitable SI including 518 µg/ml, 5.6 µg/ml, 6.36 µg/ml for *A. persica*, *A. spicigera*, and *A. fragrance* at 48 h post infection, respectively. SI estimation elucidated that all three *Artemisia* extracts were actively selective against *L. major* promastigotes, compared to macrophages with an SI of 10.1µg/ml, 2.8 µg/ml, and 1.75 µg/ml for *A. persica*, *A. spicigera*, and *A. fragrance* at 48 h post infection, respectively. Hence, *Artemisia* extracts were strongly active against *L. major* promastigotes and amastigotes in comparison with alone cell line macrophages.

Discussion

Currently available drugs for treatment of leishmaniasis are pentavalent antimony derivatives which have different side effects and almost all of the anti-leishmanial chemical compounds have various obstacles to treat the infection (39). Du to no harmful effects of herbal plants on the host cells, they can be applied as potential alternatives in the development of new anti-leishmanial agents. These herbals have selective actions against parasites without reduction of host cell viability (36, 40). Therefore, one of these herbal plants is *Artemisia*, which can be apply as novel natural compounds. Several studies have been proposed that *Artemisia* spp. may have biological functions for various clinical applications (23, 41-43).

This is the first report on comparison of in–vitro anti-leishmanial activity of Iranian Flora *A. persica*, *A. spicigera*, and *A. fragrance* ethanolic extract and against *L. major* in cell culture model. In the current study, we calculated IC50, CC50, and EC50 of three species of *Artemisia* extracts in order to specify their anti-leishmanial activities. The achieved data exhibited that studied *Artemisia* extracts have dose-dependent anti-leishmanial effects and had the inhibitory functions on the growth of *L. major*. In addition, these herbal plants are identified as anti-leishmanial agent against both form of parasite including extracellular promastigotes and intracellular amastigotes, while they have no harmful side effects for the host macrophages. The finding of the current study illustrated no toxicity in mice macrophages with even high dose of *Artemisia* extracts, which confirms its lowest harmful effects. According to published reports, other species of *Artemisia* such A. *campestris*, *A. herba-alba* Asso and *A. aucheri* plants were promising candidates as antileishmanial products which proved to be effective compounds against viability and proliferation of both promastigotes and amastigotes of *Leishmania* (44, 45). Their findings support our results. Furthermore, the antileishmanial activities of other *Artemisia* species were also described. *A. absinthium* from Cuba was able to clear *L. amazonensis* promastigotes with 50% inhibitory concentration (46). *A. annua*, was also potent against *L. donovani* (47). *A. absinthium* from Ethiopia has been documented to exhibit activity against the promastigote of both *L. aethiopica* and *L. donovani* strains forms (48). All the mentioned experiments are in line with the current findings.

Among these three ethanolic *Artemisia* extracts, *A .persica* showed the most potent leishmanicidal activity (IC50: 51 µg/ml) while, *A. fragrance* extract had less activity against *L. major* promastigotes (IC50: 400 µg/ml) after 48 h. The finding are in agreement with other studies. For instance, other *Artemisia* species such as *A. kulbadica* and *A. ciniformis* revealed the good antileishmanial effects (49). An in vitro study similar to our study was conducted to examine the effect of *A. absinthium* extract on the growth of *L. major* promastigotes. The results indicated that after 48 h of exposure, the IC50 of *A. absinthium* extract was 56 mg/ml and the leishmanicidal effect of concentrations greater than 200 mg/ml almost 80% (50). The results indicated that the optimal concentrations of the *A. spicigera* and *A. persica* extracts for reducing the amastigotes growth inside the macrophages were 100µg/ml that killed more than half of amastigotes forms. The current study has indicated that the SI values for three *Artemisia* extracts were approximately under 10, that these compounds had ideal anti-leishmanial activity and toxic for *L. major* promastigotes and amastigotes forms, in comparison with the host cell.

The leishmanicidal activities of various herbal plants such as artemisinin, racemoside, curcumin, *Piper betle* and *Aloe vera* have been documented to be interceded by apoptosis (47). *Leishmania* as an intracellular parasite can undergo programmed cell death in response to

herbal natural products (47, 51). We found that, *Artemisia* spp. had effective leishmanicidal activity as evidenced by shrinkage in the *Leishmania* promastigotes cell wall that became round in shape and had no motility with disrupted flagella, which has also been seen in the previous study (47).

Conclusion

We reported antileishmanial activities of three species of *Artemisia* plants including *A. persica*, *A. spicigera*, and *A. fragrance*, which are growing in Iran. All of them exhibited potent inhibitory and antiparasitic activity against *L. major* promastigotes and amastigotes. This is the first experiment to our knowledge that describes strong antileishmanial activity of *Artemisia* against *L. major*. Further studies are needed to identify the various bioactive compound(s) of mentioned *Artemisia* species with a view to investigate them as potential alternative herbal plants to the available drugs for treatment leishmaniasis.

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Conflict of interest

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the current manuscript.

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